Culture of IMR90 Cells in Advanced RPMI

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Method Article

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Abstract

This protocol describes the thawing, culturing, and cryopreservation of the human lung fibroblast cell line, IMR90, in Advanced RPMI.

Disclaimer: The contents of this article have been reviewed by the US Environmental Protection Agency and approved for publication and do not necessarily represent Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendations for use.

NOTE: There is a consolidated PDF version of this protocol in the supplementary files section below.

Introduction

Reagents

Reagents

1. Advanced RPMI 1640 Medium without L-glutamine & HEPES (ThermoFisher #12633-012; hereafter referred to as “basal medium”)
2. Fetal bovine serum (ThermoFisher #16000-044)
3. GlutaMAX™ supplement (ThermoFisher #35050-061)
4. 100X penicillin/streptomycin solution (ThermoFisher #15140-122)
5. Dulbecco’s phosphate buffered saline (DPBS, 1X, ThermoFisher #14190-144)
6. 0.25% Trypsin-EDTA (ThermoFisher #25200-056)
7. Dimethylsulfoxide (DMSO, Sigma #D8418)
8. Isopropanol (Sigma #I9516)
9. Trypan Blue solution (0.4%, Sigma #T8154)

Equipment

Equipment/Supplies

1. Biosafety cabinet

2. Humidified tissue culture incubator with 5% CO_2

3. Water bath

4. Collagen coated tissue culture dishes prepared using 50 µg/mL collagen concentration according to McNabb and McCullough (2019)

a. 100mm dishes (TPP #93100)

b. 150mm dishes (TPP #93150)

5. Pipet aid

6. Serological pipettes

7. 50 mL conical tubes

8. Sterile 150 mL and 250 mL bottles

9. Tabletop centrifuge

10. Eppendorf tubes (autoclaved; USA Scientific #1615-5500)

11. Pipettes

12. Filter tips (low retention)

13. Hemocytometer

14. PVDF membrane syringe filter with 0.22µm pore (Millipore #SLGVR33RS)

15. “Mr. Frosty” cell freezing apparatus (Nalgene; Fisher #5100-0001)

16. Long-term cell storage container with liquid nitrogen

Procedure

IMR90 cells are not known to be hazardous; however, they were derived from human samples and should be handled under BSL-2 precautions. Researchers should wear appropriate personal protective equipment during the completion of this protocol. A face shield and thermal gloves should be worn while handling liquid nitrogen and cell vials that have been recently removed from liquid nitrogen storage as they may explode unexpectedly.

IMR90 cells should be thawed and sub-cultured on collagen coated tissue culture plastics. IMR90 cells used within an APD range of 3 – 15 have high levels of reproducibility within sub-cultured experiments.

Quality Control:

Cells will be visually observed for culture quality prior to every passage. There should be an average of 1.25 (± 0.37 SD) population doublings observed per passage under these conditions. Cultures should be subjected to short tandem repeat (STR) profiling with first use and periodically thereafter to identify potential cell line contaminants. Cultures should be tested for the presence of the intracellular bacterial pathogen *Mycoplasma* spp. At least every three months

Prepare Growth Medium

1. Remove 37.5 mL of volume from the bottle of basal medium using a 50 mL pipet and discard.

2. Add 25 mL of fetal bovine serum (FBS) to the remaining volume using a 25 mL pipet.

a. Final FBS concentration: 5%

3. Add 2.5 mL of 100X penicillin/streptomycin using a 5 mL pipet.

a. Final Pen/Strep concentration: 0.5%

4. Add 10 mL of GlutaMAX supplement to the medium using a 10 mL pipet.

a. Final GlutaMAX concentration: 4 mM

5. Mix thoroughly and label with the date and additives.

Thawing Cryopreserved Cells

1. Pre-warm growth medium in a 37°C water bath for at least 30 minutes and prepare materials prior to obtaining a vial of cells from cryostorage.

a. This reduces the amount of time cells are in the presence of DMSO during thawing.

2. Remove vial from liquid nitrogen and thaw in 37 °C water bath (1-2 min).

a. It is important that cells are thawed quickly to prevent damage and reduced viability.

b. Submerge to the level of the medium within the cryovial. Submerging past the junction of the tube and cap can result in contamination.

c. Once the vial has thawed, dry it with a paper towel and sanitize it by spraying with 70% ethanol.

3. Add 24 mL of pre-warmed growth medium to a 50 mL conical tube with a 25 mL pipet. Then, transfer 1 mL of the thawed cells from the cryovial into the growth medium with a P1000 pipette and gently mix by inversion.

4. Pellet cells via centrifugation at 1,000 x *g* for 4 minutes at room temperature.

5. Carefully aspirate the supernatant.

6. Add 25 mL of warmed growth medium to the cell pellet using a 25 mL pipet and gently resuspend the cells by triturating three times. Then transfer the cell suspension to a collagen-coated 15 cm tissue culture dish.

7. Distribute the cells evenly by gentle rocking and swirling for at least 10 seconds.

8. Incubate in a humidified 37 °C incubator with 5% CO_2 at ambient O_2 concentration overnight.

9. Check cells the following day for attachment to the dish. Aspirate medium to remove dead cells (rounded, unattached cells). Replace with 25 mL of pre-warmed growth medium using a 25 mL pipet. It is preferable to culture thawed cells for at least two passages before using in experiments.

Sub-Culturing Cells

NOTE: Cells should be split three days after being plated at 4.5 x 10^4 cells/mL. See appendices for representative images of cells ready to be split after three days in culture. Waiting more than three days between passages and/or plating fewer cells will adversely affect reproducibility in downstream experiments.

1. Pre-warm growth medium and DPBS in a 37°C water bath and a trypsin aliquot at room temperature for at least 30 minutes.

2. Sanitize biosafety cabinet working surface by spraying a paper towel with Cavicide then wiping the working area. Then spray the working surface 70% ethanol and wipe until dry.

3. Transfer plates of cells being passaged from the incubator to the biosafety cabinet.

4. Aspirate the cell culture medium.

5. Gently add warmed DPBS to each plate and rinse by swirling carefully.

a. 20 mL for 15 cm dishes (25 mL pipet)

b. 10 mL for 10 cm dishes (10 mL pipet)

6. Aspirate the DPBS wash.

7. Add trypsin and rotate/rock to spread evenly across the surface of the dish.

a. 1.5 mL for 15 cm dishes (5 mL pipet)

b. 750 µL for 10 cm dishes (P1000 pipette)

8. Place in tissue culture incubator for 3 minutes (time varies by trypsin lot and will need to be determined empirically for each lot). To ensure uniform heating of the bottom surface of each plate, do not stack dishes on top of each other in the incubator during this step. Following incubation, use the palm of your hand to gently hit the side of the dish to facilitate detachment. Check detachment with microscope before proceeding.

a. **NOTE**: If cells are still attached, incubate for another 2 minutes. Incubate and tap dish until >95% of the cells are detached.

9. Add 12 mL of growth medium and with a 10 mL pipet and gently rock/swirl to cover the surface of the dish with the medium. Triturate once to break up any large cell clumps, swirl to mix, and transfer the cell suspension to a 50 mL conical tube.

10. Wash the plate by adding 12 mL of additional growth medium with a 10 mL pipet. Then gently rock, triturate, and add to the cell suspension that was collected in in Step #9.

a. **NOTE**: Typically, 12 mL of medium is used to wash and collect remaining cells per 1-2 15 cm plates trypsinized. If using two 15 cm plates, the plates can be serially washed with the same volume of medium prior to combining with the cell suspension from Step #9.

11. Centrifuge at 1,000 x *g* for 4 minutes to pellet cells.

12. Carefully aspirate the supernatant and resuspend the cell pellet in 12 mL of growth medium using a 10 mL pipet. Using the same pipet, triturate four times to thoroughly break up the majority of clumped cells. Add additional growth medium to dilute the cell suspension if desired.

a. **NOTE**: Typically, medium is added such that there is a total volume of 12 mL per 1-2 15 cm plates collected.

13. Prepare a 1:2 dilution of 50 µL of the cell suspension in a 1.5 mL Eppendorf tube by mixing 50 µL of Trypan Blue using a P200 pipette. Mix by vortexing for 1-2 seconds.

14. Transfer 10 µL of the Trypan Blue-treated diluted cell suspension to each side of a hemocytometer using a P20 pipette. Count and record the number of cells that exclude Trypan Blue (*i.e.*, intact cells) in the four corner grids on each side of the hemacytometer

a. **NOTE**: Do not use an automated cell counter as these cells display variability in size and are not accurately quantified by automated methods).

b. **NOTE**: Blue staining of cells indicates a damaged membrane. Blue-stained cells are considered to be non-viable.

15. Calculate the number of population doublings that occurred during the last passage using the following formula (include the indicated values in the associated worksheet):

([3.32(log(cells collected per plate)-log(cells plated per plate)) + previous APD])

16. Prepare a plating suspension at 4.5 x 10^4 cells/mL in growth medium in a 50 mL tube or larger sterile bottle to the total volume that will be used for plating (10 mL for each 10 cm dish and 25 mL for each 15 cm dish, making 2-5 mL extra).

a. If larger volumes are required, then they can be prepared in sterile disposable bottles. Mix cell suspensions in bottles by swirling for 10-20 seconds instead of inversion.

17. Mix thoroughly by inversion and dispense the diluted cell suspension into each collagen coated dish using a 10 mL or 25 mL pipet for 10mL or 25 mL volume, respectively.

a. **NOTE**: Cells spread out more evenly when collagen coated dishes are pre-warmed to 37°C.

18. Incubate in a humidified incubator at 37°C with 5% CO_2 and ambient O_2 concentration. Cells will be ready to split three days after plating.

a. **NOTE:** Plating cells at a determined cell density will result in more predictable sub-culture and more reproducible performance in subsequent experimental assays.

19. Place bottles of medium and DPBS in 5% CO_2 incubator with lids partially unscrewed for 15-30 minutes to adjust pH by re-gassing before returning to 4 °C storage.

Preparing Cells for Cryopreservation

1. Prepare freezing medium.

a. 50% FBS, 40% growth medium, 10% DMSO.

b. Filter through a 0.22 µm pore syringe filter.

2. Follow sub-culturing protocol through Step #15.

3. Calculate and record the total number of viable cells in the cell suspension.

4. Pellet cells by centrifugation at 1,000 x *g* for 4 minutes at room temperature.

5. Resuspend the cell pellet to a cell density of 1.25 x 10^6 cells/mL in freezing medium by gently triturating three times.

6. Transfer 1 mL of the cell suspension per cryovial using a P1000 pipette.

7. Put vials in a Mr. Frosty controlled freezing container (filled to indicated line with isopropyl alcohol) and place in a -80 °C freezer overnight.

a. The chamber regulates cooling to about 1 °C per minute.

b. **NOTE:** Isopropanol should be changed after every three freezing cycles.

c. **NOTE:** Do not leave vials at -80 °C longer than overnight as it will impact viability after thawing.

8. On the following morning, transfer the frozen vials to liquid nitrogen storage.

References

McNabb, N. and McCullough, S. (2019) Collagen Coating for Tissue Culture. *Protocol Exchange*.

Figures

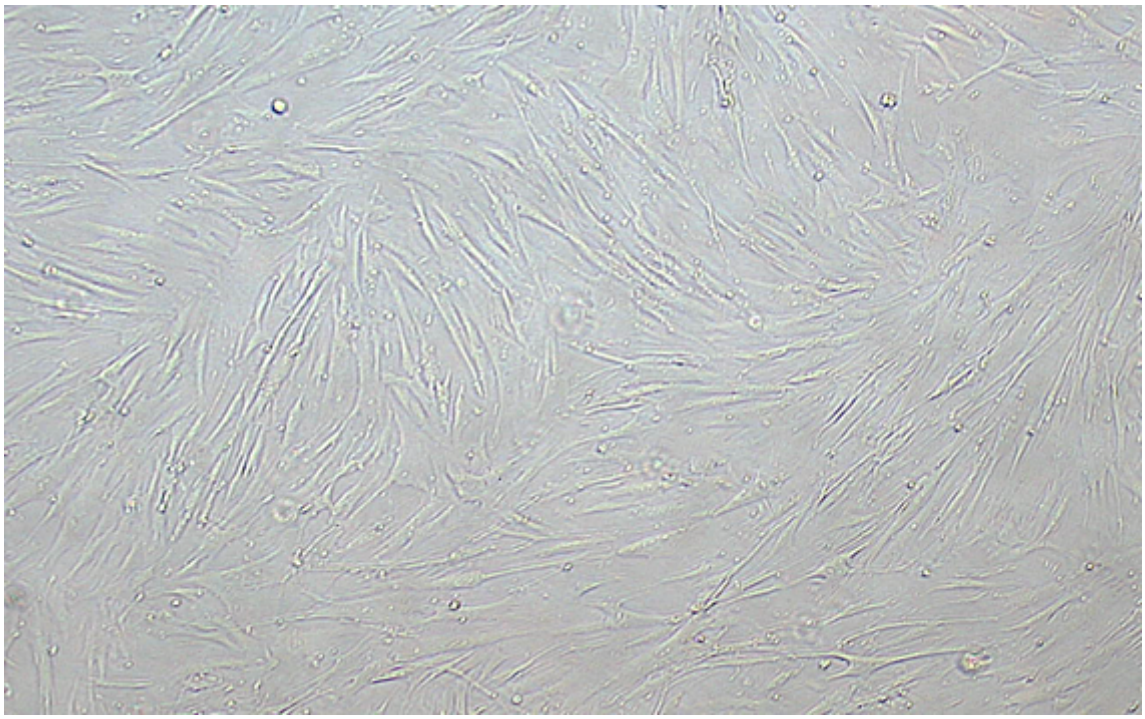

Figure 1

IMR90 cell density before splitting on day 3.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- CultureofIMR90CellsinARMPI.docx